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Highly Potent Antimicrobial Peptide Derivatives of Bovine Cateslytin

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T. M. Postma,[a] and R. M. J. Liskamp[a]*

Antimicrobial peptides are potentially valuable antibiotics. More research is required to turn these compounds into viable therapeutics. Herein, a novel highly potent antimicrobial peptide is presented as a tool to study antimicrobial peptides. Antibacterial activity in the low micromolar range, low haemolytic activity and excellent serum stability were observed.

Antibiotics form one of the pillars upon which modern medicine stands.[1] This position has become threatened by the rapid rise of multidrug-resistant bacteria. In some clinical cases bacteria were identified that have become resistant to all known antibiotics, which is a serious global public health threat.[2,3] There is an urgent need for new antibiotics with novel mechanisms that especially can be used to treat the clinically more relevant Gram-negative bacteria. Antimicrobial peptides (AMPs) are a first line of defence against microbial infection in humans and many other organisms.[4,5] They are typically positively charged and therefore strongly interact with negatively charged bacterial membranes causing perturbation or pore formation.[6–8] AMPs likely differentiate between bacterial and eukaryotic cells largely based on the difference in membrane composition. Bacterial membranes predominantly contain zwitterionic phosphatidylethanolamine together with up to 25% negatively charged phospholipids such as phosphatidylglycerol.[9] Conversely, eukaryotic cells contain cholesterol and mostly phosphatidylcholine lipids, an essentially neutral zwitterionic outer membrane monolayer.[10] The mechanism of AMPs is very different from typical small molecule antibiotics and less prone to the induction of antimicrobial resistance.[11] Moreover, the most attractive feature of AMPs is that they can circumvent antimicrobial resistance to small molecules by acting on the cell membrane, which can hardly be altered under the selection pressure of an antibiotic.[12] Given the ubiquitous nature of AMPs in mammals and other organisms, there is high potential for an optimized peptide therapeutic to treat bacterial infections and more importantly infections of multidrug-resistant bacteria.[13] However, AMPs have not yet been successful as therapeutics and require more research to enable the design of high-potential AMP drug candidates. This is partially caused by the challenge that a "membrane" as a target poses, as every cell has a membrane. Therefore, optimizing selectivity has become an important aspect to avoid any toxic side effects by antibiotic peptides. Herein, we report a novel highly potent and non-haemolytic peptide sequence as a new tool to study AMPs.

The parent sequence used in this study is based on the mammalian antimicrobial peptide cateslytin, part of the innate immune system, which is released by proteolytic cleavage from the protein chromatogranin A upon microbial infection.[14,15] Chromogranin A can function as a pro-hormone with several bioactive peptide cleavage products that cover a wide range of functions in the cardiovascular, endocrine and immune systems.[16] Chromogranin A is released from chromaffin cells upon stimulation of the adrenal gland or polymorphonuclear neutrophils.[17] Cateslytin, a short 15 amino acid peptide, demonstrated activity against a range of Gram-positive and Gram-negative bacteria, yeasts and fungi.[18] Dufourc and coworkers studied the interaction of cateslytin with bacterial and mammalian membrane mimics, which was found to act by a different mechanism compared to most AMPs.[19,20] Cateslytin is unstructured in solution and forms antiparallel β–sheets upon interaction with a negatively charged bacterial membrane. The cateslytin antiparallel β–sheets did not form pores but aggregated in negatively charged membranes to promote rigid domains that can disrupt membrane integrity leading to cell death. It is likely that this results in the selectivity of cateslytin for negatively charged bacterial cells over zwitterionic phospholipid containing more neutral membranes of mammalian cells.

Bovine cateslytin (RSMRLSFRARGYGFR) was significantly more potent than the human analogue (SSMKLSFRARGYGF) as

Table 1: Synthesized antimicrobial peptides

Peptide	Amino Acid Sequence

[a] School of Chemistry, Joseph Black Building, University of Glasgow, University Avenue, Glasgow, G12 8QQ, United Kingdom.
* Email corresponding author: robert.liskamp@glasgow.ac.uk
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1 (cateslytin)	H-RSMRLSFRARGYGFR-OH
2	H-RSMRLSFRARGYGFR-NH_2
3	H-RSL*RLSFRARGYGFR-OH
4	H-RSL*RLSFRARGYGFR- NH_2
5	H-RSL*RLSWRARGYGFR- NH_2
6	H-RSL*RLSWRARGYGWR- NH_2
7 (synlytin)	H-RTL*RLTWRARGYGWR- NH_2

Footnote: L* is norleucine

it contains two additional positively charged arginine residue. A series of modified short peptide sequences of 15 amino acids were prepared using Fmoc solid-phase peptide synthesis (Table 1). The sequence of bovine cateslytin (**1**) contains a C-terminal carboxylic acid which can potentially impact solubility, stability and activity. In the first cateslytin analogue the C-terminal carboxylic acid was changed into a C-terminal amide (**2**). Both cateslytin (**1**) and peptide **2** contain the amino acid methionine, which is prone to oxidation to methionine S-oxide. Typically, methionine is often not critical for the activity of a peptide and can be exchanged by the unnatural amino acid norleucine. In this analogue, the methionine thioether moiety was replaced by a methylene unit. Therefore, two peptides were prepared with norleucine and either a C-terminal carboxylic acid (**3**) or amide (**4**). In many cationic antimicrobial peptides the activity can be fine-tuned by altering the amphiphilic balance between charged and hydrophobic residues. To alter the amphiphilic properties of cateslytin several analogues were prepared. The phenylalanine residues were changed to either one (**5**) or two (**6**) tryptophan residues, a very common amino acid in antimicrobial peptides.[21] The final peptide included an additional modification in which the amino acid serine was replaced for threonine to slightly alter the amphiphilic balance by addition of extra methyl groups (**7**).[22]

Following peptide synthesis the antibacterial activity was determined against a panel of clinically relevant bacteria (Table 2). The bacteria chosen for evaluation of the peptides were the Gram-negative *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, and the Gram-positive *Staphylococcus aureus* and *Staphylococcus epidermidis*. Of these bacteria, five out of six are part of the "ESKAPE" pathogens.[23] ESKAPE pathogens are a group of bacteria that are prone to antimicrobial resistance and therefore have a high clinical priority for the development of new antibiotics. The antibacterial activity was determined in a microbroth dilution assay to determine the minimum inhibitory concentration (MIC).[24] The MIC is defined as the lowest concentration of an antibiotic that inhibits visible growth of bacteria as observed with the unaided eye.

Cateslytin (**1**) showed inhibition of bacterial growth with MIC values in the range of 4 to 32 µM for all Gram-negative and Gram-positive bacteria, with the exception of *S. aureus*. The MIC for *S. aureus* was higher than 128 µM. Changing the C-terminal carboxylic acid of cateslytin (**1**) into a C-terminal amide in peptide **2** resulted in a 2-fold increased potency for all bacteria except *S. aureus*. In peptide **3** methionine was replaced for norleucine, which caused a doubling in potency from cateslytin (**1**) and roughly the same as peptide **2**. Peptide **4** contained a C-terminal amide together with a norleucine residue that doubled the potency of peptide **3**. However, the MIC for *S. aureus* stayed above 128 µM. Changing one of the phenylalanine residues into a tryptophan in combination with norleucine and a C-terminal amide significantly increased the potency for *S. aureus* in peptide **5** to an MIC of 32 µM. By changing both phenylalanine residues to tryptophan in peptide **6** the potency increased 2-fold compared to peptide **5**, except for *S. aureus*. The pronounced increase in potency after

Table 2: Evaluation of peptide MIC values against Gram-negative and Gram-positive bacteria (µM)

Bacterium	**Gram**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**Ampicillin**
Escherichia coli	-	4	2	2	1	1	1	0.5	11
Klebsiella pneumoniae	-	8	2	4	2	4	2	1	168
Pseudomonas aeruginosa	-	32	8	8	4	8	4	2	21
Acinetobacter baumannii	-	32	16	16	8	16	8	4	5
Staphylococcus aureus	+	>128	>128	>128	>128	32	32	16	337
Staphylococcus epidermidis	+	32	4	8	2	4	2	1	84

introducing tryptophan residues may be largely due to the following. Cateslytin and derivatives are rich in arginine with 5 amino acid residues in a 15-peptide. As many antimicrobial peptides are remarkably rich in both arginine and tryptophan residues, the phenylalanine residues were replaced by tryptophan.[25] The indole sidechain of tryptophan may interact with the cationic guanidino group of arginine. These groups can form a parallel cation - π interaction in which arginine retains the ability to hydrogen bond with other molecules.[26] Of course many cation - π interactions can take place between cationic and aromatic amino acids, but tryptophan offers a much larger and stronger region of negative electrostatic potential, which would make tryptophan overall a better cation binding site than phenylalanine or tyrosine.[27] Furthermore, this interaction shields cationic arginine residues from the hydrophobic environment inside the lipid bilayer and may facilitate membrane entry by an antimicrobial peptide.[28] These properties of both arginine and tryptophan containing antimicrobial peptides may be responsible for the increased antibacterial activity observed with peptides **5** and **6**. A similar potentiation was observed with a tryptophan and arginine containing 15 residue lactoferricin derivatives, where a substitution for tryptophan in position 8 resulted in a 6-fold increase in antibacterial activity against *E. coli*.[29] An additional modification, in which the serine residues were replaced with threonine, resulted in the most potent peptide (**7**) in this series. Overall, the amphiphilic balance of cateslytin was gradually shifted to an increased hydrophobicity by the modifications. Increasing hydrophobicity in an antimicrobial peptide may result in more potent antibacterial activity although this can greatly increase haemolysis.[30] Therefore, the serine residues were changed to threonine as this only gave a mild increase in hydrophobicity. Peptide **7** demonstrated potent inhibition of bacterial growth with MIC values between 0.5 and 16 µM for all bacteria tested. With an MIC value of 16 µM against *S. aureus* peptide **7** was significantly more potent than the parent sequence cateslytin (**1**) with an MIC of >128 µM against this strain. Altering the amphiphilic balance of cateslytin by several modifications in the peptide sequence resulted in a very potent antimicrobial peptide that was active against both Gram-negative and Gram-positive bacteria. All peptides were significantly more potent than the antibiotic ampicillin, which was used as a control.

Table 4: Percentage haemolysis at 128 µM

Peptide	Percentage haemolysis
1 (cateslytin)	0 ± 0.1
2	1.1 ± 0.4
3	n.d.
4	0.3 ± 0.1
5	0.4 ± 0.4
6	0.4 ± 0.1
7 (synlytin)	0.3 ± 0.1

Footnote: n.d. is not determined

To further demonstrate the utility of the most potent synthetic cateslytin analogue **7**, referred to as "synlytin", it was compared to cateslytin (**1**) against several drug resistant bacteria. Two strains of *A. baumannii* were used with mutations in LpxA (AL1851) and LpxD (AL1852).[31,32] Mutations in the LpxA/D acetyltransferases halt early lipid A biosynthesis, which results in a lipopolysaccharide deficiency that causes resistance against polymyxin E. Two drug resistant strains of *P. aeruginosa* with high resistance against β–lactam antibiotics were used. The final strain used to demonstrate activity against resistant bacteria was a β–lactam resistant strain of *Pandoraea apista*, an emerging pathogen in respiratory infections.[33] The MIC values of peptides **1** and **7** against these resistant bacteria were determined (Table 3).

Cateslytin (**1**) inhibited growth at 8 and 4 µM for the lipopolysaccharide deficient *A. baumannii* strains. Synlytin (**7**) was 4-fold more potent than cateslytin (**1**) and inhibited growth of *A. baumannii* strains at 2 and 1 µM. In both *P. aeruginosa* strains, cateslytin (**1**) did not inhibit growth within

Table 3: Evaluation of peptide MIC values against drug-resistant bacteria (µM)

Bacterium	Gram	1	7	Polymyxin E	Ampicillin
A. baumannii (AL1851)	-	8	2	3	3
A. baumannii (AL1852)	-	4	1	>400	3
P.aeruginosa (TP161)	-	>128	8	0.2	>1346
P.aeruginosa (TP162)	-	>128	4	0.2	>1346
P. apista (TP163)	-	8	0.5	6	>1346

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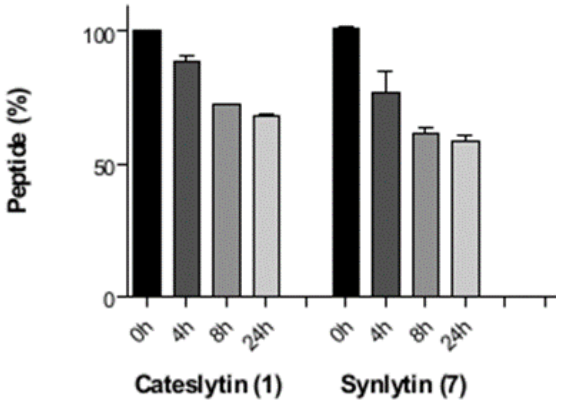

Figure 1: Serum stability of cateslytin (**1**) and synlytin (**7**)

the concentration range tested with an MIC >128 µM. In contrast, synlytin (**7**) inhibited the growth of the *P. aeruginosa* strains at 8 and 4 µM. In the β–lactam resistant *P. apista* strain, cateslytin (**1**) gave an MIC of 8 µM, whereas synlytin (**7**) was 16 times more potent with an MIC of 0.5 µM. There results demonstrate that synlytin (**7**) is a significantly more potent antimicrobial peptide than cateslytin (**1**).

Antimicrobial peptides may show severe cytotoxicity to red blood cells. This toxicity is caused by the amphiphilic nature of most cationic peptides, which can act as potent cationic detergents. Lysis of red blood cells is an unacceptable form of toxicity in antimicrobial peptides and therefore must be quantified in a haemolytic assay. A wide range of haemolytic tendencies in studied antimicrobial peptides has been observed such as the highly toxic non-selective peptides mastoparan B (96% haemolysis at 30 µM) and melittin (100% haemolysis at 10 µM) or less toxic peptides that are more selective to bacterial cells like magainin 2 (50% haemolysis at 428 µM) and HHC10 (only 0.4% haemolysis at 15.6 µg/mL).[34–37] The haemolytic activity of peptides **1**-**7** was determined using a haemolytic assay developed in our group with minor modifications (Table 4).[37] In this assay sheep erythrocytes were used as a convenient source of red blood cells.

All evaluated peptides showed very little haemolysis even at 128 µM, which is 8 – 256 times higher than the MICs for the evaluated bacteria, when compared to 100% haemolysis (1% Triton X-100). At this concentration most peptides caused less than 1% haemolysis. These results clearly show that all cateslytin derivatives are not haemolytic even at a high concentration.

Peptides can be very unstable in human serum due to degradation by proteases. Therefore, the serum stability of cateslytin (**1**) and the most potent peptide synlytin (**7**) was determined by a method used previously in our group.[38] Human serum was used to evaluate the stability of the peptides for up to 24h at 37 °C (Figure 1).

Both cateslytin (**1**) and synlytin (**7**) showed excellent serum stability, cateslytin demonstrated a slightly better stability, with over 60% of the peptides remaining after 24h. Cateslytin was reported to be stable to *Staphylococcus aureus* proteases by Metz-Boutigue and co-workers.[15] The proteolytic stability of cateslytin (**1**) and synlytin (**7**) may be explained by cateslytin itself, since it is the final cleavage product of extensive proteolytic processing of chromogranin A and therefore it is probably less sensitive to further proteolytic degradation.

Conclusions

A series of bovine cateslyin derivatives were synthesized and the biological activity was evaluated. All peptides showed antimicrobial activity against Gram-negative and Gram-positive bacteria in the micromolar range. Synlytin (**7**) demonstrated the highest antimicrobial activity against a panel of clinically relevant bacteria. Cateslytin (**1**) and synlytin (**7**) were evaluated against several drug resistant bacteria, in which synlytin (**7**) was found significantly more potent than cateslytin (**1**). Cateslytin (**1**) and synlytin (**7**) demonstrated low haemolytic activity at even high concentration and excellent serum stability. These results clearly show that synlytin (**7**) is a superior AMP compared to the parent cateslytin (**1**) in terms of potency against Gram-negative, Gram-positive and drug-resistant bacteria while remaining non-haemolytic and serum stable.

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